AD_____

Award Number: W81XWH-04-1-0795

TITLE: Effects of Hematopoietic Lineage and Precursor Age on CML Disease Progression

PRINCIPAL INVESTIGATOR: Kenneth Dorshkind, Ph.D.

CONTRACTING ORGANIZATION: University of California, Los Angeles
Los Angeles, CA 90095

REPORT DATE: March 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-03-2007	Final	1 Sep 2004 – 28 Feb 2007

4. TITLE AND SUBTITLE

Effects of Hematopoietic Lineage and Precursor Age on CML Disease Progression

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-04-1-0795

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

Kenneth Dorshkind, Ph.D.

Email: kdorshki@mednet.ucla.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of California, Los Angeles
Los Angeles, CA 90095

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
Original contains colored plates: ALL DTIC reproductions will be in black and white.

14. ABSTRACT

Declines in lymphopoiesis during aging diminish the immune response, but little consideration has been given to its effect on the development of hematological disease. This report demonstrates that age-related defects in lymphopoiesis contribute to the myeloid dominance of adult leukemia. Using a murine model of chronic myeloid leukemia (CML), an adult-onset malignancy arising from transformation of hematopoietic stem cells (HSC) by the BCR-ABLP210 oncogene, our studies have demonstrated that young bone marrow (BM) cells transformed with BCR-ABLP210 initiated both a myeloproliferative disorder (MPD) and B lymphoid leukemia while BCR-ABLP210 transformed old BM cells recapitulated the human disease by inducing a MPD with rare lymphoid involvement. Further, the lesser severity of MPDs initiated from old BCR-ABLP210 transduced BM cells revealed unappreciated defects in aged myeloid progenitors. These data demonstrate the influence of aging on patterns of leukemogenesis and indicate that its effects on hematopoiesis are more widespread than has been appreciated.

15. SUBJECT TERMS
Hematopoietic Stem Cells, CML, Leukemia

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
U	U	U	UU	41	USAMRMC

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

INTRODUCTION

Aging is associated with declines in lymphocyte production, and this effect has largely been considered in the context of its impact on the quality of the adaptive immune response, which is diminished in the elderly. Less attention has been given to how these age-related alterations influence disease within the hematopoietic system, and hematopoietic malignancies in particular. For example, the majority of leukemias that present in children involve lymphoid cells, and these occur at a time when lymphoid progenitor number and proliferation are highest. Conversely, myeloid leukemias tend to predominate in the elderly when lymphopoiesis is waning. Chronic myeloid leukemia (CML) provides a case in point. Despite the fact that CML is a hematopoietic stem cell disease, CML primarily presents as a myeloproliferative disease with infrequent lymphoid involvement. This disease pattern, combined with the fact that CML is a disease of middle and old age, has led us to hypothesize that the age-related decline in lymphopoiesis is a factor that influences the myeloid predominance of this and other adult-onset leukemias. In order to test this premise, we used CML as a model system and examined the pattern of disease induced by BCR-ABL^{P210} transformation of bone marrow cells from young and old mice. Our results indicate that the development of myeloid and lymphoid leukemias is directly related to the age of hematopoietic stem and progenitor cells, and we are currently interrogating various signaling pathways in young and old hematopoietic stem/progenitors to determine why this is the case.

BODY

CML presents primarily as a myeloid hyperplasia in patients in both its acute and chronic phases. When malignant lymphoid cells are observed, they are primarily B lymphoid lineage in origin. This overwhelming presentation of myeloid cell dominance in CML is puzzling given that the chromosomal translocation in CML occurs in hematopoietic stem cells (1-9). In view of these points, the objective of this proposal was to address how does aging affects CML progression. Aging is known to result in a diminution of lymphopoiesis. Because CML is primarily a disease of middle to old age, we hypothesized that the predominant myeloid and relatively infrequent lymphoid involvement was due to the age-associated declines in lymhopoiesis .

These questions were addressed by performing the following tasks:

Task 1: *Enrich lineage negative (Lin-) c-kit^{hi} Sca-1^{hi} HSC, Lin- Sca-1- c-kit+ CD127 (IL-7Rα)- Common Myeloid Progenitors (CMP) and Lin- Sca-1^{lo} c-kit^{lo} CD127 (IL-7Rα)+ Common Lymphoid Progenitors (CLP) from young and old BALB/c mice;*

Our laboratory developed the procedures to label bone marrow cells with antibodies to multiple cell surface determinants and to isolate various hematopoietic progenitor populations by flow cytometry. We developed expertise in the isolation of HSC, common lymphoid progenitors (CLP), common myeloid progenitors (CMP), and committed lymphoid and myeloid progenitors.

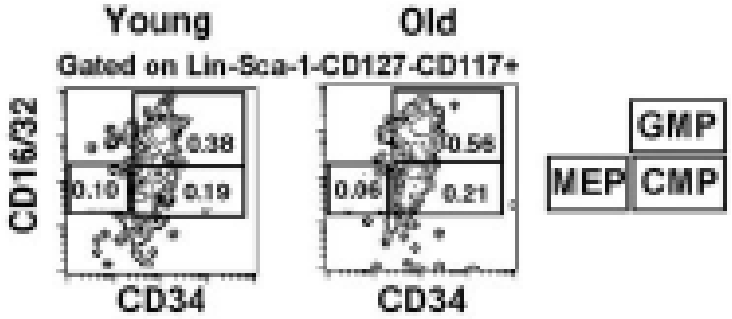

Figure 1. Resolution of megakaryocyte-erythroid (MEP), Granulocyte-Macrophage (GMP), and common myeloid progenitors (CMP) by FACS analysis. Figure from reference 10.

Task 2: *Culture cells in medium, cytokine cocktail (IL-3, IL-6, c-kit ligand for HSC and CMP and IL-3, IL-6, c-kit ligand, IL-7 for CLP), and retroviral vector containing either 5' long terminal repeat (LTR)-driven BCR-ABL^{P210} internal ribosome entry site (IRES) enhanced green fluorescent protein (EGFP) or 5' LTR-driven IRES EGFP for 24-36 hours;*

We received the BCR-ABL construct from our collaborator, Dr. Owen Witte. This gene was then inserted into a murine retrovirus. We then prepared stocks of retrovirus to be used for transduction of hematopoietic cells. A particular issue with some transduction protocols is that they involve the culture of purified hematopoietic stem cells with retrovirus for several days, and in our original application we indicated that our incubation time would be between 24-36 hours. However, a problem with this approach is that the stem cells may have differentiated by this time. Therefore, we developed a methodology to incubate bone marrow cells with retrovirus plus cytokines for only six hours as follows:

Young mice were administered a single intravenous dose of 5-Fluorouracil (5-FU, 150 mg/kg body weight for young mice, Sigma). Due to their higher susceptibility to 5-FU treatment, middle-age and old mice were given a 5-FU dose of 115 mg/kg body weight. On the eighth day after 5-FU administration, BM cell suspensions were prepared by flushing femurs and tibias with Ca^{2+} and Mg^{2+}-free PBS (PBS). Following red blood cell lysis with Tris–ammonium chloride, pH 7.2, 4-5 x 10^6 cells were distributed in 5 ml polystyrene tubes (Becton Dickinson) and incubated with 1 ml of retrovirus supplemented with 10% horse serum (Hyclone), 1mM L-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin, 8 μg/ml polybrene (Sigma), 50 μM β-mercaptoethanol (Sigma), 25 mM HEPES (Gibco), 100 ng/ml SCF (Biosource), 100 ng/ml Flt-3L (R&D), and 10 ng/ml IL-11 (R&D) for 2 hours at 37 ºC in 5% CO_2 and air and constant humidity. Cells were then centrifuged for 5 minutes at 400*g*, and viral supernatant was replaced with 1 ml of fresh virus stock supplemented as described above. This procedure was repeated twice. After a total of 6 hours, cells were washed, counted, and resuspended in PBS. Infection efficiency of young and old BM cells was tested in colony assays and was found to be comparable. This protocol is described and used in our *Blood* manuscript (11) that is currently under review and which is appended to this report.

Task 3*: Inject low (10^5) and high (10^6) doses of transduced cells into groups of six lethally irradiated, syngeneic recipients;*

We have repopulated several groups of mice with BCR-ABL transduced bone marrow cells from young and old mice. As shown in Figure 2, they develop either myeloproliferative disease (MPD), B lymphoproliferative disease, or a combination of the two. These mice have developed leukemia, and they were analyzed as described below.

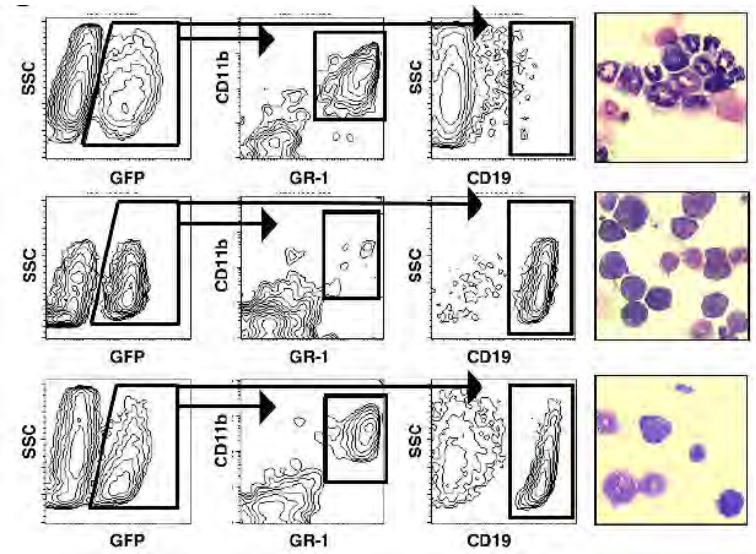

Figure 2. A. BM from young or old B6 mice was harvested on day 8 following 5-FU treatment, infected with a retrovirus carrying IRES and BCR-ABL and a reporter EGFP gene and transplanted into sublethally irradiated, young syngeneic recipients.. Examples of recipients with myeloproliferative disease (top), B lymphoid leukemia (middle), and both (bottom) are shown. Images are from figure 1 in a manuscript submitted to *Blood (11)*.

Task 4: Analyze *peripheral blood at 2 weeks post-transplantation for morphology and cell phenotype;*

The submitted application indicated that we would analyze peripheral blood of mice two weeks following the transplantation of BCR-ABL infected bone marrow into them. However, we have found that mice reliably develop leukemia at 4-8 weeks following transplantation. Therefore, we have not performed the peripheral blood analysis at earlier time points. Instead, as described in Task 5 we performed a detailed phenotypic analysis of bone marrow and spleen of mice that developed leukemia.

Task 5: *Sacrifice mice when animals appear diseased, perform histopathologic and phenotypic analysis on GFP+ (donor derived) and GFP- (endogenous host) bone marrow, spleen, lymph nodes, thymus, and peripheral blood cells in all the animals. In addition, confirm integration of BCR-ABL by PCR and Southern blotting;*

We have now processed 20 mice that were repopulated with young, BCR-ABL transduced bone marrow and 15 mice that received a transplant of old BCR-ABL infected bone marrow cells. Because the retrovirus in which the BCR-ABL gene was inserted is bi-cistronic and contains the gene encoding green fluorescence protein (GFP) in addition to BCR-ABL, we were able to assess the repopulation in the myeloid ($CD11b^+$ and $Gr\text{-}1^+$), erythroid ($Ter119^+$), B lymphoid ($B220^+$), and T ($CD4^+$ and $CD8^+$) lineages as shown in Figure 3 from our submitted *Blood* manuscript depict the types of leukemias that arise from BCR-ABL transduced young and old bone marrow cells .

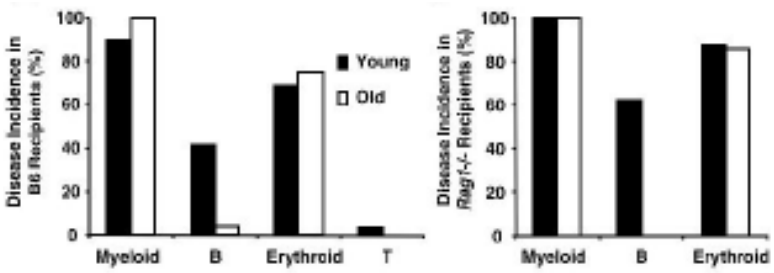

Figure 3. Incidence of myeloid, B, erythroid, and T lineages leukemias in B6 (right) and RAG-/- recipients of young and old BCR-ABL transduced BM. Adapted from a manuscript under review in *Blood (11).*

Task 6*:* *Analyze data and determine how the lineage and age of the progenitor influences disease progression.*

We analyzed the mice that received young, BCR-ABL transduced cells and the mice that received old transduced cells. The results clearly demonstrated that the degree of B lineage involvement with disease was related to the age of the transduced cells (Figure 4). The data are striking in that the mice that received young cells developed leukemias that were both myeloid and lymphoid in their presentation. In contrast, mice that received old cells developed leukemias, but these were almost exclusively myeloid in their presentation.

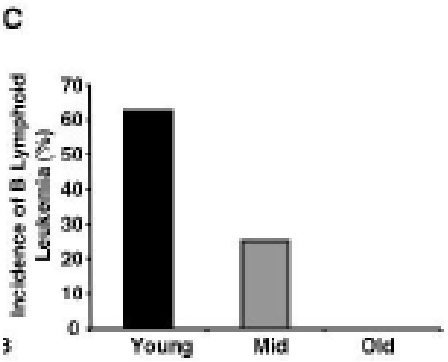

Figure 4. Incidence of lymphoid leukemia in recipients of young (3 month old), middle age (10-12 months old), and old (18-22 months old) BCR-ABL transduced bone marrow cells. Figure adapted from manuscript under review in *Blood (11).*

.

To our knowledge, this will be the first report to link changes in lymphopoiesis with aging as a factor that explains the presentation of CML.

KEY RESEARCH ACCOMPLISHMENTS

- Developed methodologies to purifiy hematopoietic progenitor populations and to infect them with retroviral vectors containing the BCR-ABL gene

- Demonstrated that the pattern of BCR-ABL mediated leukemia is influenced by the age of the hematopoietic targets

- The analysis of patterns of leukemogenesis in mice repopulated with BCR-ABL transduced cells unexpectedly revealed that age-related defects are also manifest in myeloid progenitors.

REPORTABLE OUTCOMES

- An abstract describing this work was presented at the 4th International Society for Stem Cell Research meeting in Toronto in June 2006.
- An abstract describing this work was presented at the Keystone Symposium on Cancer Stem Cells in March 2007
- A manuscript describing this work has been submitted and is currently under review at *Blood*.
- The award supported in part the doctoral thesis work of Robert Signer, a graduate student enrolled in the Cellular and Molecular Pathology Graduate Program at UCLA.

CONCLUSION

The importance of this work is that it provides an understanding of why CML presents clinically as a predominantly myeloid disease. Specifically, the minor lymphoid involvement reflects the fact that the lymphoid developmental potential of aged hematopoietic stem and progenitor cell populations is severely compromised. In addition, as detailed in the attached *Blood* manuscript, these studies led to the demonstration of myeloid defects in aging, which had heretofore not been appreciated.

These results suggest a next step, which is to identify specific reasons why lymphoid developmental potential of aged hematopoietic cells is compromised. In this regard, we are now extending these studies to interrogate signaling pathways responsible for the patterns of leukemogenesis observed in young and old mice.

REFERENCES

1. Enright, H. and P. McGlave. Chronic myelogenous leukemia. In Hematology: Basic Principles and Practice, 3rd edition. R. Hoffman, E.J. Benz, Jr., S. J. Shattil, B. Furie, H.J. Cohen, L.E. Silberstein, and P. McGlave, eds. Churchill Livingstone, New York.

2. Delforge, M., M.A. Boogaerts, P.B. McGlave, and C.M. Verfaillie. 1999. BCR/ABL-CD34+HLA-DR- progenitor cells in early chronic phase, but not in more advanced phases, of chronic myelogenous leukemia are polyclonal. Blood 93:284-292.

3. Savage, D.G., R.M. Szydlo, and J. M. Goldman. 1997. Clinical features at diagnosis in 430 patients with chronic myeloid leukemia seen at a referral center over a 16-year period. Br. J. Haematol. 96:111-116.

4. Gunz, F.W. 1977. The epidemiology and genetics of the chronic leukemias. Clin. Hematol. 6:3-20.

5. Brincker, H. 1982. Population-based age- and sex-specific incidence rates in the four main types of leukemia. Scand. J. Haematol. 29:241-249.

6. Wertheim, J.A., J.P. Miller, L. Xu, Y. He, and W.S. Pear. 2002. The biology of chronic myelogenous leukemia: mouse models and cell adhesion. Oncogene. 21:8612-8628.

7. Kantarjian, H.M., M. J. Keating, M. Talpaz, et al. 1987. Chronic myelogenous leukemia in blast crisis. Analysis of 242 patients. Am. J. Med. 83:445-454.

8. Kabarowski, J.H.S. and O.N. Witte. 2000. Consequences of BCR-ABL expression within the hematopoietic stem cell in chronic myeloid leukemia. Stem Cells. 18:399-408.

9. Raskind, W.H. and P.J. Fialkow. 1987. The use of cell markers in the study of human hematopoietic neoplasia. Adv. Cancer Res. 49:127-167.

10. Min, H., E. Montecino-Rodriquez, and K. Dorshkind. 2006. Effect of ageing on the common lymphoid progenitor to pro-B cell transition. J. Immunol. 176:1007-1012.

11. Signer, R.A., E. Montecino-Rodriguez, O. Witte, J. McLaughlin, and K. Dorshkind. Effects of aging on CML disease progression. Blood. Submitted.

APPENDICES

1. *Blood* manuscript (under review).
2. Abstract: 4th International Society for Stem Cell Research meeting
3. Abstract: Keystone Symposium

SUPPORTING DATA

None

FINAL REPORT LIST OF PERSONNEL RECEIVING PAY FROM RESEARCH EFFORT

Robert A.J. Signer

FINAL REPORT BIBLIOGRAPHY

1. Signer, R.A.J., E. Montecino-Rodriguez, and K. Dorshkind. Aging alters cancer stem cell developmental potential in chronic myeloid leukemia. 4th International Society for Stem Cell Biology Meeting Abstract.

Chronic Myeloid Leukemia (CML) is an adult onset myeloproliferative disorder (MPD) arising from transformation of hematopoietic stem cells (HSC) by the BCR-ABL^{P210} fusion oncogene. Despite originating in HSC, BCR-ABL^{P210} induced leukemia presents predominantly as CML and rarely causes lymphoid disease. We determined that age-associated declines in lymphopoiesis underlie this myeloid biased disease presentation. Using a murine model, we demonstrate that young bone marrow (BM) cells transformed with BCR-ABL^{P210} initiate both MPDs and lymphoid leukemia, while BCR-ABL^{P210} transformed old BM cells recapitulate the human disease by inducing MPDs with rare lymphoid involvement. We further document that BCR-ABL^{P210} induced MPDs derived from old BM cells are characterized by a reduced tumor burden when compared to those derived from young BM cells and show this is related to heretofore unappreciated developmental defects present in aged myeloid progenitors. Furthermore, transplantation experiments indicate that leukemia stem cells generated from BCR-ABL^{P210} transformed old BM cells exhibit these age-related lympho- and myelopoietic defects. Taken together, our results provide a biological explanation for both the myeloid dominance and chronic nature of human CML and demonstrate the impact of senescence and stem cell aging on the development of hematopoietic malignancies.

This work was supported by grants from the National Institutes of Health (AG-21459) and the U.S. Department of Defense (W81XWHO410795). R.A.J.S. is supported by a fellowship from the California Institute for Regenerative Medicine (TI-00005).

2. Signer, R.A.J., E. Montecino-Rodriguez, O.N. Witte, J. McLaughlin, and K. Dorshkind. 2007. **A**ge-related hematopoietic defects underlie the myeloid dominance of adult leukemia. Keystone Symposium Cancer Stem Cells Meeting

Chronic Myeloid Leukemia (CML) is an adult onset myeloproliferative disorder (MPD) arising from transformation of hematopoietic stem cells (HSC) by the BCR-ABL^{P210} fusion oncogene. Despite originating in HSC, BCR-ABL^{P210} induced leukemia presents predominantly as CML and rarely causes lymphoid disease. We determined that age-associated declines in lymphopoiesis underlie this myeloid biased disease presentation. Using a murine model, we demonstrate that young bone marrow (BM) cells transformed with BCR-ABL^{P210} initiate both MPDs and lymphoid leukemia, while BCR-ABL^{P210} transformed old BM cells recapitulate the human disease by inducing MPDs with rare lymphoid involvement. We further document that BCR-ABL^{P210} induced MPDs derived from old BM cells are characterized by a reduced tumor burden when compared to those derived from young BM cells and show this is related to heretofore unappreciated developmental defects present in aged myeloid progenitors. Furthermore, transplantation experiments indicate that leukemia stem cells generated from BCR-ABL^{P210} transformed old BM cells exhibit these age-related lympho- and myelopoietic defects. Taken together, our results provide a biological explanation for both the myeloid dominance of CML and other adult leukemias, and demonstrate the impact of senescence and stem cell aging on the development of hematopoietic malignancies.

This work was supported by grants from the National Institutes of Health (AG-21459) and the U.S. Department of Defense (W81XWHO410795). R.A.J.S. is supported by a fellowship from the California Institute for Regenerative Medicine (TI-00005).

4. Signer, R.A., E. Montecino-Rodriguez, O. Witte, J. McLaughlin, and K. Dorshkind. Effects of aging on CML disease progression. Blood. Submitted

PDF of document attached as an appendix.

Age-related defects in B lymphopoiesis underlie the myeloid dominance of adult leukemia

Robert A.J. Signer[1], Encarnacion Montecino-Rodriguez[1], Owen N. Witte[2,3,4], Jami McLaughlin[2], Kenneth Dorshkind[1]

[1] Department of Pathology and Laboratory Medicine and the Hematopoietic Malignancies Program, Jonsson Comprehensive Cancer Center, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.

[2] Department of Microbiology, Immunology and Molecular Genetics, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.

[3] Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, University of California, Los Angeles, CA 90095, USA.

[4] Howard Hughes Medical Institute, University of California, Los Angeles, CA 90095, USA.

Corresponding author: Kenneth Dorshkind
Department of Pathology and Laboratory Medicine and the Hematopoietic Malignancies Program
Jonsson Comprehensive Cancer Center
David Geffen School of Medicine
University of California, Los Angeles
10833 Le Conte Avenue
Los Angeles, CA 90095
USA
Tel.: 1-310-206-9535
Fax: 1-310-206-9391
E-mail: kdorshki@mednet.ucla.edu

Scientific Category: Hematopoiesis

Total Word Count: 4987

Abstract Word Count: 150

Running Title: Effects of aging on leukemogenesis

Key Words: aging, BCR-ABL, CML, hematopoiesis, hematopoietic stem cells, leukemia, leukemia stem cells, lymphopoiesis, myelopoiesis, senescence.

Abstract

Declines in lymphopoiesis during aging diminish the immune response, but little consideration has been given to its effect on the development of hematological disease. This report demonstrates that age-related defects in lymphopoiesis underlie the myeloid dominance of adult leukemia. Using a murine model of chronic myeloid leukemia (CML), an adult-onset malignancy arising from transformation of hematopoietic stem cells (HSC) by the BCR-ABL^{P210} oncogene, we demonstrate that young bone marrow (BM) cells transformed with BCR-ABL^{P210} initiated both a myeloproliferative disorder (MPD) and B lymphoid leukemia while BCR-ABL^{P210} transformed old BM cells recapitulated the human disease by inducing a MPD with rare lymphoid involvement. Further, the lesser severity of MPDs initiated from old BCR-ABL^{P210} transduced BM cells revealed unappreciated defects in aged myeloid progenitors. These data demonstrate the influence of aging on patterns of leukemogenesis and indicate that the effects of senescence on hematopoiesis are more extensive than has been previously appreciated.

Introduction

Hematopoietic stem cells (HSC) are the precursors from which all mature blood cells are generated[1,2]. HSC normally maintain the hematopoietic system through balanced differentiation into common myeloid (CMP) and lymphoid specified progenitors. CMP, the earliest myeloid specified progenitors to be defined, subsequently differentiate into granulocyte-macrophage (GMP) and megakaryocyte-erythroid progenitors (MEP) from which most myeloid and erythroid cells respectively arise[3]. While there is uncertainty regarding the characteristics of the most HSC proximal lymphoid specified progenitors[4], there is general agreement that common lymphoid progenitors (CLP)[5] are a canonical intermediate through which B cell development progresses. The progeny of CLP include pre-pro-B and pro-B cells, and upon successful rearrangement of immunoglobulin (Ig) heavy chain genes, pre-B cells that express immunoglobulin (Ig) μ heavy chain in their cytoplasm are produced. Surface IgM^+ B cells are generated from pre-B cells following rearrangement and expression of Ig light chain genes[6,7].

Recent analyses have shown that the balance of hematopoietic cell production becomes severely perturbed during aging. B lymphocyte development begins to decline early in adult life and the production of B cells is dramatically diminished in aged individuals[8,9]. This decline is manifest at all stages of B cell development, as both the frequency and number of CLP and their downstream pre-pro-B, pro-B, and pre-B cell progeny are significantly reduced with age[10-14]. Since HSC accumulate multiple functional defects with increasing age[15-18], it has been proposed that the decline in B lymphopoiesis is the result of age-related deficiencies in the potential of HSC to generate lymphoid progeny[19,20] as well as to proliferative and differentiative defects intrinsic to lymphoid intermediates[9,21]. Despite increasing evidence that aged HSC do not proliferate and differentiate as efficiently as their young counterparts[22], myelopoiesis has been reported to be unaffected by aging[11,19]. This conclusion is based on the finding that the frequency of CMP and their downstream progeny remains normal or is increased in old mice.

The consequences of HSC aging and reduced lymphocyte production have, for the most part, been considered in the context of their impact on the quality of the adaptive immune response, which is diminished in the elderly[23]. Less attention has been given to

how these age-related alterations influence disease within the hematopoietic system, and hematopoietic malignancies in particular[24]. For instance, the majority of leukemias that present in children involve lymphoid cells, and these occur at a time when lymphoid progenitor number and proliferation are highest[8,11]. Conversely, myeloid leukemias tend to predominate in the elderly when lymphopoiesis is waning[19].

CML, the most common MPD in humans, typifies this pattern[25]. CML presents as a myeloid hyperplasia that occurs almost exclusively in adults and its incidence increases with age. Over 90% of CML patients possess the Philadelphia chromosome[26,27], a reciprocal translocation t(9q34;22q11)[28] that fuses the breakpoint cluster region (*BCR*) and Abelson tyrosine kinase (*ABL*) genes[29-31] and encodes a 210 kDa chimeric protein with constitutive tyrosine kinase activity[30,32]. The occurrence of the BCR-ABL^{P210} translocation in HSC[33-36] has led to CML being classified as a disease of stem cell origin[37]. Since HSC have multilineage differentiation potential[38], their transformation would be expected to result in disease with proportionate representation of both the myeloid and lymphoid lineages. However, this is not the case, since BCR-ABL^{P210} induced leukemia predominantly presents as CML and rarely causes disease with lymphoid involvement.

This disease pattern, combined with the fact that CML is a disease of middle and old age[39], led us to hypothesize that the age-related decline in lymphopoiesis is a factor that contributes to the myeloid predominance of this and other adult-onset leukemias. In order to test this premise, we used CML as a model system and examined the pattern of disease induced by BCR-ABL^{P210} transformation of BM cells from young and old mice. The data support the validity of our hypothesis and further reveal that, in contrast to what has been generally accepted, myelopoiesis is also compromised by aging. Taken together, this study demonstrates that immune senescence contributes to the myeloid dominance of adult leukemia and indicates that the effects of aging on blood cell development are more extensive than is currently appreciated.

Materials and Methods

Mice

Four to 7 week old C57BL/6J (B6) and B6.129S7-Rag1^{tm1Mom}/J (*Rag1*$^{-/-}$) mice were purchased from The Jackson Laboratory, and B6.SJL mice were obtained from Taconic Farms. Ten to 24 month old B6 mice were purchased from the National Institute on Aging colony. Animals were housed in the vivarium of the University of California at Los Angeles Division of Laboratory Animal Medicine. Animal care and experiments were conducted according to the guidelines of the Institutional Animal Care and Use Committee of the University of California at Los Angeles.

B6, B6.SJL, and *Rag1*$^{-/-}$ recipients were preconditioned with 500 R 12-30 hours before intravenous injection of transformed cells from a ^{137}Cs irradiator (120 R/min; Mark I-68A; JL Shepperd and Associates).

Generation of retroviral stocks

The retroviral vector pMSCV[40] containing a 5' LTR-driven BCR-ABL internal ribosome entry site (IRES) enhanced GFP (EGFP) or a 5' LTR-driven IRES EGFP was used to generate high-titer helper-free retrovirus stocks following transient cotransfection of 293T cells. 293T cells were grown at 50-70% confluence on Poly-L-Lysine (Sigma) coated 10 cm tissue culture treated plates (Becton Dickinson) in Iscove's modified Dulbecco's minimum essential medium (IMDM, Mediatech) supplemented with 10% fetal calf serum (FCS, Hyclone), 1mM L-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin (complete IMDM; all from Gibco). Transfections were done by co-precipitating 15 μg of retroviral vector with 15 μg of an ecotropic packaging vector[41] in a total volume of 1 ml of Ca-PO_4 and 2x HEPES Buffered Saline from the CalPhos Mammalian Transfection Kit (BD Biosciences). The precipitate was then added to 293T cells in 9 ml of culture media. The cells were then incubated at 37 ºC and 5% CO_2 and air and constant humidity. Medium was replaced after 12, 24, 36, 48 and 60 hours with 10, 5, 5, and 5 mL of complete IMDM, respectively. Viral stocks were prepared by pooling the supernatants collected at 36, 48, and 60 hours post-transfection. Viral titers were determined following infection of 3T3 cells with serial dilutions of the pooled virus supernatant. Viral titers

were found to range between 2 x 10^6 and 7 x 10^6 virus particles/ml. Viral stocks were then stored in 2 ml aliquots at -80 ºC until use.

Retroviral transduction and bone marrow transplantation

Young mice were administered a single intravenous dose of 5-Fluorouracil (5-FU, 150 mg/kg body weight for young mice, Sigma). Due to their higher susceptibility to 5-FU treatment, middle-age and old mice were given a 5-FU dose of 115 mg/kg body weight. On the eighth day after 5-FU administration, BM cell suspensions were prepared by flushing femurs and tibias with Ca^{2+} and Mg^{2+}-free PBS (PBS). Following red blood cell lysis with Tris–ammonium chloride, pH 7.2, 4-5 x 10^6 cells were distributed in 5 ml polystyrene tubes (Becton Dickinson) and incubated with 1 ml of retrovirus supplemented with 10% horse serum (Hyclone), 1mM L-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin, 8 μg/ml polybrene (Sigma), 50 μM β-mercaptoethanol (Sigma), 25 mM HEPES (Gibco), 100 ng/ml SCF (Biosource), 100 ng/ml Flt-3L (R&D), and 10 ng/ml IL-11 (R&D) for 2 hours at 37 ºC in 5% CO_2 and air and constant humidity. Cells were centrifuged for 5 minutes at 400*g*, and viral supernatant was replaced with 1 ml of virus stock supplemented as described above. This procedure was repeated twice. After 6 hours, cells were washed, counted, and resuspended in PBS. Infection efficiency of young and old BM cells was tested in colony assays and was found to be comparable. Pre-conditioned 5 to 7 week old B6, B6.SJL, or *Rag1*$^{-/-}$ mice were then injected intravenously with 2 x 10^5 cells per mouse. For secondary transplants 5 x 10^6 splenocytes from primary diseased mice were injected intravenously into preconditioned 5-7 week old B6 recipients. All cell suspensions were counted with a hemocytometer, and viability was determined by eosin dye exclusion.

Immunophenotypic analysis of leukemic cells and cell sorting

BM cell suspensions were prepared as described above. Spleen cell suspensions were prepared by dissociation between frosted slides. Following red blood cell lysis, cell suspensions were incubated with anti-CD16/32 (FcγRII-III; clone 2.4G2; eBiosciences) or total mouse IgG (for CMP and GMP stains only; Axell) to reduce nonspecific labeling. Cells were washed and incubated with combinations of antibodies to the following cell

surface determinants, conjugated to fluoro-isothiocyanate, phycoerythrin, tricolor, indodicarbocyanine, biotin, or allophycocyanin: CD3ε (clone KT31.1), CD4 (clone GK1.5), CD8α (clone 53-6.7), CD11b (clone M1/70), CD16/32 (FcγRII/III; clone 2.4G2), CD19 (clone 1D3), CD45.1 (clone A20), CD45.2 (clone 104), CD45R (B220, clone RA3-6B2), CD117 (c-Kit, clone 2B8), CD127 (IL-7Rα, clone A7R34),Ter119 (clone Ter-119), TCRβ (clone H57-597), TCRγδ (clone UC7-13D5), NK1.1 (clone PK136), Ly-6C (clone AL-21), AA4.1 (clone C1qRp), Sca-1 (clone E13-161.7), IgM (clone II/41), and Gr-1 (clone RB6-8C5). Biotinylated cells were visualized by incubation with tricolor-, allophycocyanin-, or allophycocyanin-Alexa Fluor 750- conjugated streptavidin. All reagents were obtained from Becton Dickinson or eBiosciences except for goat anti–mouse IgM (Southern Biotech), tricolor-conjugated CD11b and allophycocyanin- Alexa Fluor 750– conjugated streptavidin (Caltag Laboratories). Optimum working dilutions were determined for each antibody before use. All incubations were for 20 – 40 minutes at 4°C in PBS. After the last wash, live cells were acquired with Cell Quest Software (Becton Dickinson) on a FACScan, FACSCalibur or Cytek modified FACSscan (all BD Biosciences) located at the Flow Cytometry Core of Jonsson Comprehensive Cancer Center at the University of California at Los Angeles.

Disease phenotype was established by determining the frequencies of BM or spleen cells co-expressing GFP (BCR-ABL^{P210}) and lineage specific cell surface antigens (Myeloid: CD11b^{+}Gr-1^{+}, B lymphoid: CD45R^{+}CD19^{+}, Erythroid: Ter119^{+}, T-Lymphoid: CD4^{+} and/or CD8^{+}). Disease was considered present when at least 7.5% of the total GFP^{+} cells in either the BM or spleen co-expressed the specific cell surface antigens.

Populations enriched for CMP[3] were defined as Lin^{-} (Lin = CD3ε, CD8α, CD45R, Gr-1, TER-119, TCRαβ, and TCRγδ) Sca-1^{-}CD127^{-}CD16/32$^{+/Lo}$CD117^{Hi}. Populations enriched for GMP[3] were defined as Lin^{-} (Lin = CD3ε, CD8α, CD45R, Gr-1, TER-119, TCRαβ, and TCRγδ) Sca-1^{-}CD127^{-}CD16/32^{Hi}CD117^{Hi}. Populations enriched for HSC[38] were defined as Lin^{-} (Lin = CD3ε, CD8α, CD11b, CD45R, Gr-1, TER-119, TCRαβ, and TCRγδ) Sca-1^{Hi}CD117^{Hi}. Pre-pro B cells[42] were defined as Lin^{-} (Lin = CD3ε, CD4, CD8α, CD11b, Gr-1, TER-119, Ly-6C, IgM, TCRαβ, and TCRγδ) CD45R^{+}CD19^{-}AA4.1^{+} and Pro/Pre B cells[42] were defined as Lin^{-} (Lin = CD3ε, CD4,

CD8α, CD11b, Gr-1, TER-119, Ly-6C, IgM, TCRαβ, and TCRγδ) $CD45R^{+}CD19^{+}AA4.1^{+}$.

Cells to be purified were resuspended in minimum essential medium-α (αMEM, Gibco) supplemented with 2% FCS, 25 mM Hepes, 1mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, and 50 μg/ml gentamycin (Sigma) before being sorted on a FACSaria (Becton Dickinson). The purity of the sorted fractions was determined by reanalysis. Samples were routinely 95% pure.

B cell cultures

BM cells from day 8 5-FU treated mice were retrovirally transduced as described above. 3×10^5 cells were then seeded onto pre-established confluent S17 stromal cells[43] in T12.5 flasks (Becton Dickinson) in RPMI 1640 (Gibco) supplemented with 5% FCS, 50 μM β-mercaptoethanol, 1 mM L-glutamine, 100 U/ml streptomycin, and 100 μg/ml penicillin[44]. Cultures were incubated at 37 ºC in 5% CO_2 and air and constant humidity, and fed twice weekly for three weeks. GFP^{+} B cell production in the cultures was assessed by immunostaining as described above.

Myeloid cultures

Methylcellulose colony assays were performed by resuspending splenocytes from diseased $BM^{BCR\text{-}ABL}$ recipients or whole BM cells or purified CMP from young and old mice in methylcellulose supplemented with 30% FCS, 40% αMEM, 50 μM β-mercaptoethanol, 1 mM L-glutamine, 100 U/ml streptomycin, 100 μg/ml penicillin, 50 μg/ml gentamycin, 20 ng/ml SCF, 10 ng/ml GM-CSF (Biosource), 30 ng/ml IL-3 (Biosource), and 10 ng/ml IL-11[3]. Cells were plated in triplicate in 1 ml of supplemented methylcellulose in 3.5 cm petri dishes (Becton Dickinson) at a density of 5×10^4 BM or spleen cells or 250 CMP/ml. Petri dishes were placed in humidification plates and incubated at 37 ºC in 5% CO_2 and air. On day 8 colonies were counted, and individual colonies were picked under a dissecting microscope and resuspended in 0.5 ml PBS. Cells were then washed in 1.5 ml PBS and centrifuged at 400*g* for 5 minutes. Supernatant was discarded, and the cells were resuspended in 300 μl of PBS. Cells were then acquired on a FACScan for 30 seconds at a fixed flow rate of 60 μl/min. Live cells were gated, and

total cell numbers were calculated as [total live events/(flow rate*acquisition time)]*sample volume.

For myeloid liquid cultures, 1.5 x 10^5 whole BM cells or 2 x 10^3 sorted CMP isolated from young or old mice were plated in 3-6 wells each in 12 well plates in 1.5 ml of complete IMDM supplemented with 50 μM β-mercaptoethanol, 50 μg/ml gentamycin, 20 ng/ml SCF, 10 ng/ml GM-CSF, 30 ng/ml IL-3, 30 ng/ml IL-6 (Biosource), and 10 ng/ml IL-11. Cultures were incubated at 37 ºC in 5% CO_2 and air and constant humidity. On day 4 cultures were fed with 0.5 ml of supplementd IMDM as described above. On day 6 cultures were harvested by collecting the supernatant and trypsinization of adherent cells. Cells were counted with a hemocytometer. Myeloid cell production was assessed by immunostaining with Gr-1 and CD11b as described above.

Statistical analysis

Unless indicated otherwise, data are expressed as a mean ± SEM. Differences between groups were tested by a two-tailed, unpaired t-test, with an α of 0.05.

Results

Hematopoietic cell age alters BCR-ABL^{P210} induced leukemia phenotype

To assess the impact of aging on BCR-ABL^{P210} induced leukemogenesis[45], BM cells harvested from day 8 5-fluorouracil (5-FU) treated young (5-7 weeks) or old (90-104 weeks) mice were infected with a retrovirus carrying a bicistronic IRES expression vector encoding BCR-ABL^{P210} and an EGFP reporter gene and subsequently injected into sublethally irradiated syngeneic young mice (BM$^{BCR-ABL}$ recipients; Figure 1A). Control animals were similarly transplanted with BM cells infected with a retrovirus containing EGFP alone (BM^{EGFP} recipients). Congenic CD45.1$^+$ recipients were used in one experiment and confirmed that GFP$^+$ (BCR-ABL$^+$) leukemic cells co-expressed the donor derived CD45.2 cell surface antigen (data not shown).

Recipients of both young and old BM$^{BCR-ABL}$ cells exhibited weight loss, cachexia, and poor grooming and were sacrificed between 2 to 7 weeks post-transplantation. These animals had decreased BM and increased splenic cellularity compared to BM^{EGFP} recipients (Figure 1B and 1C). Hematopoietic infiltration of the

liver and lungs with occasional lymphadenopathy was evident at necropsy, and symptoms appeared more severe in young $BM^{BCR\text{-}ABL}$ recipients (data not shown). In contrast, all BM^{EGFP} recipients were observed for up to four months post-transplantation and remained healthy.

Disease patterns in $BM^{BCR\text{-}ABL}$ recipients were assessed by phenotypic and morphologic characterization of hematopoietic cells in their BM and spleen (Figure 1D and Table 1). Ninety percent (26/29) of young and 100% (24/24) of old $BM^{BCR\text{-}ABL}$ recipients developed MPDs characterized by the expansion of $GFP^+Gr\text{-}1^+CD11b^+$ granulocytes in the BM and spleen that was frequently accompanied by the expansion of $GFP^+Ter119^+$ erythroid cells (Figure 1D and 1E). Similar to the situation in humans, 96% (23/24) of old $BM^{BCR\text{-}ABL}$ recipients lacked significant B lineage involvement in their disease, as determined by the minimal frequency of $GFP^+CD45R^+CD19^+$ B lineage cells and absence of lymphoid blasts in their BM and spleen. In stark contrast, 35% (9/26) of young $BM^{BCR\text{-}ABL}$ recipients that developed MPDs concurrently developed B lymphoid leukemia. In addition, 10% (3/29) of young $BM^{BCR\text{-}ABL}$ recipients presented with B lymphoid leukemia without significant myeloid involvement, a disease profile that was never observed in any old $BM^{BCR\text{-}ABL}$ recipients.

Overall, 41% (12/29) of young $BM^{BCR\text{-}ABL}$ recipients developed B lymphoid leukemia compared with 4% (1/24) of old $BM^{BCR\text{-}ABL}$ recipients (Figure 1E). This was not a result of decreased homing[18], since increasing the number of old $BM^{BCR\text{-}ABL}$ cells transplanted did not induce lymphoid disease (data not shown). Therefore, these data strongly indicate that $BCR\text{-}ABL^{P210}$ transduced old hematopoietic cells lack significant potential to initiate B lymphoid leukemia.

This conclusion was confirmed by transplanting $BCR\text{-}ABL^{P210}$ transduced young and old BM cells into *Rag1*$^{-/-}$ recipient mice, whose lack of lymphocytes creates a more favorable environment for normal and dysplastic lymphoid development[46-48]. As expected, while all $BM^{BCR\text{-}ABL}$ *Rag1*$^{-/-}$ recipients developed MPDs, young $BM^{BCR\text{-}ABL}$ recipients developed B lymphoid leukemia with an incidence markedly higher (63%; 5/8) than wild type recipients. In contrast, none (0/7) of the old $BM^{BCR\text{-}ABL}$ *Rag1*$^{-/-}$ recipients showed any significant B lineage component to their disease (Figure 1F).

The incidence of B lymphoid leukemia correlates with age-related declines in B lymphopoiesis

The age-related decline in B lymphopoiesis does not abruptly initiate in old age, but instead begins in relatively young animals and progresses gradually thereafter[8]. For example, middle-age mice (42-44 weeks) have approximately half the number of B lineage cells as young mice (Figure 2A and 2B). Similarly, the frequency of pre-pro-B, pro-B, and pre-B cells is progressively reduced in the BM of 5-FU treated mice of increasing age, demonstrating a reduced capacity to generate B lineage cells *de novo* in old mice[11].

If the age-related defects in B cell production underlie the reduced capacity of $BM^{BCR\text{-}ABL}$ cells to initiate B lymphoid leukemia, then the incidence of $BCR\text{-}ABL^{P210}$ induced lymphoid disease should decline in parallel with the age-associated loss of B cell developmental potential. This hypothesis was tested by transplanting $BCR\text{-}ABL^{P210}$ transduced BM cells from young, middle-age, and old mice into *Rag1*$^{-/-}$ recipients. As shown in Figure 2C, the incidence of B lymphoid leukemia is highest amongst young $BM^{BCR\text{-}ABL}$ recipients (63%; 5/8), intermediate in middle-age $BM^{BCR\text{-}ABL}$ recipients (25%; 2/8), and nil in recipients of old $BM^{BCR\text{-}ABL}$ (0%; 0/7). Therefore, as hypothesized, the B lymphoid leukemogenic potential of $BCR\text{-}ABL^{P210}$ transduced BM cells closely reflects the capacity of hematopoietic cells to generate B lymphoid cells at the time of transformation.

In agreement with these data, we also observed that $BCR\text{-}ABL^{P210}$ confers a growth advantage to B lineage cells from young but not old mice when grown in B lymphoid permissive conditions *in vitro*[44]. The total number of B lineage cells harvested from cultures initiated with young $BM^{BCR\text{-}ABL}$ cells was approximately 70 fold higher than from cultures initiated with young BM^{EGFP} cells (Figure 2D). However, the number of B lineage cells harvested from cultures established with old $BM^{BCR\text{-}ABL}$ cells was only 2-3 fold higher than from cultures initiated with old BM^{EGFP} cells and was approximately 100 fold lower than from cultures initiated with young $BM^{BCR\text{-}ABL}$ (Figure 2D). Visual inspection confirmed that, in contrast to old $BM^{BCR\text{-}ABL}$ cells, young $BM^{BCR\text{-}ABL}$ cells established abundant lymphoid-like colonies (Figure 2E), and flow cytometric analyses

showed that few $GFP^+CD19^+CD45R^+$ B lineage cells were present in cultures established with old $BM^{BCR\text{-}ABL}$ cells (Figure 2E).

These observations indicate that $BCR\text{-}ABL^{P210}$ cannot rescue the proliferative and differentiative defects responsible for the decreased B cell developmental potential of aged hematopoietic progenitors.

Decreased severity of MPDs derived from old $BM^{BCR\text{-}ABL}$

Compared to recipients of old $BM^{BCR\text{-}ABL}$ cells, young $BM^{BCR\text{-}ABL}$ recipients consistently presented with increased wasting and a more pronounced invasion of organs such as liver and lung with leukemic cells (data not shown). Although young and old $BM^{BCR\text{-}ABL}$ recipients had comparable numbers of cells in their BM and spleen (Figure 1B and 1C), the proportion of cells that were GFP^+ was higher in young $BM^{BCR\text{-}ABL}$ recipients. For example, the proportion of GFP^+ cells in the spleen of young and old $BM^{BCR\text{-}ABL}$ recipients was 62% and 52%, respectively and 47% and 29% in the BM ($P<0.002$), respectively. Since this increased tumor burden in young $BM^{BCR\text{-}ABL}$ recipients could have reflected the addition of B lymphoid leukemia to their MPDs, we compared young and old $BM^{BCR\text{-}ABL}$ recipients that developed MPDs without B lineage involvement. Surprisingly, the difference in tumor burden was accentuated in these animals due to a higher frequency of $GFP^+Gr\text{-}1^+CD11b^+$ cells (Figure 3A and 3B). This increased tumor burden in young $BM^{BCR\text{-}ABL}$ recipients resulted in increased tissue disruption demonstrated by an enhanced displacement of endogenous B lineage cells in the spleen (Figure 3C). This discrepancy in tumor burden was not the result of increased retroviral transduction efficiency of transplanted young cells, as determined by the comparable proportion of GFP^+ myeloid colonies derived from young and old BM cells plated in methylcellulose immediately following *in vitro* retroviral transduction (data not shown).

The increase in leukemic myeloid cells in young $BM^{BCR\text{-}ABL}$ recipients was accompanied by an increased frequency and number of GFP^+ HSC, common myeloid (CMP) and granulocyte-macrophage progenitors (GMP) in the BM and spleen (Figure 3D and 3E), and splenocytes from young $BM^{BCR\text{-}ABL}$ recipients formed more GFP^+ colonies than splenocytes from old $BM^{BCR\text{-}ABL}$ recipients when tested in methylcellulose myeloid colony assays (data not shown).

Identification of myelopoietic defects in old mice

The above data are surprising, because the frequency of HSC and myeloid progenitors is increased in the BM of old mice (Figure 4A)[11,19]. Despite this, preliminary analyses indicated that the colony forming activity of old BM cells was lower than would be predicted from the elevated progenitor frequency (data not shown). This was in part due to a 7-8 fold increase in the number of apoptotic Annexin V^+ CMP and GMP in old as compared to young BM (Figure 4B). Interestingly, individual colonies derived from old BM cells contained approximately 50% fewer cells than those derived from young BM cells (Figure 4C), and purified old CMP similarly generated smaller colonies than purified young CMP (Figure 4D).

This age-related reduction in myelopoietic potential was also observed when whole BM cells or sorted CMP isolated from young and old mice were grown in liquid culture supplemented with myelopoietic cytokines. Consistent with results from the colony assays, BM cells and CMP isolated from old mice produced approximately 45% less Gr-1^+CD$11b^+$ myeloid cells than those isolated from young animals (Figure 4E and 4F). Taken together, these data demonstrate that myeloid cell production by aged BM cells is diminished due to intrinsic defects in aged myeloid progenitors.

Age-related hematopoietic defects alter the potential of leukemia stem cells (LSC)

Hematopoietic stem and progenitor cells play a critical role in the pathogenesis of BCR-ABL^{P210} induced leukemia. HSC have been implicated as the leukemic cell of origin, while committed myeloid and lymphoid progenitors have been deemed the LSC in more advanced stages of disease[36,37,49]. Consequently, we assessed whether age-related differences in the pathogenesis of BCR-ABL^{P210} induced leukemia was a reflection of changes intrinsic to LSC. Since LSC are defined by their potential to transplant disease[50,51], splenocytes isolated from diseased mice grafted with young or old $BM^{BCR-ABL}$ were transplanted into sublethally irradiated syngeneic mice, and disease patterns and progression in these secondary recipients were analyzed.

Secondary recipients rapidly developed disease symptoms and were sacrificed. All these mice (27/27) presented with MPDs. However, 60% (9/15) of mice grafted with

splenocytes from young $BM^{BCR\text{-}ABL}$ recipients also presented with B lymphoid leukemia (Figure 5A). In contrast, none (0/12) of the mice grafted with splenocytes from old $BM^{BCR\text{-}ABL}$ recipients developed lymphoid disease (Figure 5B). These data suggest that either no B lymphoid LSC were produced following BCR-ABL^{P210} transduction of old hematopoietic cells, or that if they were produced, they have intrinsic defects that disrupt their leukemia initiating potential.

In addition, we compared secondary recipients that developed MPDs without B lineage involvement for disease severity. Leukemic burden was 3 fold greater in secondary recipients transplanted with young $BM^{BCR\text{-}ABL}$ derived tumors when compared with secondary recipients of old $BM^{BCR\text{-}ABL}$ derived tumors (Figure 5C and 5D). These data demonstrate that myeloid LSC derived from young $BM^{BCR\text{-}ABL}$ have a greater expansive potential through either enhanced self-renewal and/or increased production of mature leukemic myeloid progeny. Taken together, these data indicate that age-related intrinsic hematopoietic defects ultimately alter the leukemogenic potential of LSC generated following transformation of hematopoietic cells by BCR-ABL^{P210}.

Discussion

The present report has examined declines in B lymphocyte development that occur during aging in the context of hematopoietic malignancies and provides a biological explanation for the myeloid predominance of adult-onset leukemia. Although this study focused on declines in B cell development, T cell production also declines with increasing age[21], which may explain the extreme rarity with which T cell leukemia presents in older humans.

It has been proposed that changes intrinsic to aged HSC that alter their capacity to generate lymphoid progeny underlie age-related declines in B lymphopoiesis[19]. In this case, our data would be explained by the fact that BCR-ABL^{P210} transduced young HSC would have differentiated into progeny capable of developing into a MPD and B lymphoid leukemia, while aged HSC predominantly would give rise to myeloid disease. Such a scenario presumes that the leukemias described herein are derived from BCR-ABL^{P210} transduced HSC, which is a logical assumption based on reports indicating that murine BCR-ABL^{P210} induced leukemia requires HSC involvement[52], that both human

BCR-ABL^{P210+} CML and acute B-lymphoblastic leukemia (B-ALL) are derived from HSC possessing the t(9;22) translocation[33-37], and by present data showing the existence of GFP^{+} HSC in recipient mice.

Nevertheless, the possibility that leukemia initiation from the direct transformation of committed lymphoid and myeloid progenitors might also have occurred cannot be excluded, although the degree to which this may occur is unclear. With the exception of some transgenic mouse models[53,54], expression of BCR-ABL^{P210} has been shown to be insufficient to initiate leukemia in myeloid progenitors[52]. However, similar studies using lymphoid progenitors have not been as conclusive[55,56], and the possibility that they can be directly transformed by BCR-ABL^{P210} cannot be excluded. Even in this case, the reduced number of lymphoid progenitors in old BM, combined with their defects in proliferation and differentiation[9,21], would still underlie the reduced capacity to induce lymphoid leukemia following transformation. This conclusion is supported by the observation that BCR-ABL^{P210}, which significantly enhanced the growth of young B lineage cells, was unable to rescue the age-associated B lymphopoieitic growth defects of old B cell progenitors *in vitro*. Interestingly, since the expression of a potent oncogene like BCR-ABL^{P210} is not sufficient to circumvent the intrinsic growth and differentiation defects of aged lymphoid progenitors, it indicates that the intracellular defects that accompany aging fundamentally alter B lineage growth potential.

Our finding that B lymphoid leukemia was much more frequently initiated with young than old BM$^{BCR-ABL}$ is consistent with the clinical observation that the incidence of B lineage leukemia, such as B-ALL, is highest in children. That children are predisposed to lymphoid leukemia is perhaps not surprising and is consistent with results from both murine and human studies. For example, B lineage progenitors from neonates and young adults cycle at levels above their aged counterparts[11]. While this increased cycling may be necessary to fill peripheral lymphoid compartments, it could increase the chance that an aberrant genetic event could occur, particularly since developing B lineage cells possess active gene rearrangement machinery[57]. This possibility, combined with the presence of chromosomal translocations in neonatal B lineage progenitors that cause a predisposition to leukemia development[58], may increase the likelihood of transformation during early B lymphopoiesis.

The observation that MPDs initiated from young $BM^{BCR-ABL}$ cells were more aggressive than those initiated from old $BM^{BCR-ABL}$ cells was quite unexpected. This pattern led to the discovery that defects do in fact accumulate in aged myeloid progenitors. Thus, while we cannot totally exclude the possibility that less efficient engraftment of old BM cells contributed to the reduced myeloid tumor burden in recipients of old $BM^{BCR-ABL}$, it is reasonable to propose that the milder MPDs are the result of age-related defects in growth and survival that limit the expansion of myeloid progenitors. It is surprising that age-related changes in myeloid progenitors were not previously reported. However, previous studies primarily examined myeloid progenitors as a population rather than on a per-cell basis, and as a result, the cell intrinsic defects defined herein had been overlooked. The age-related defects that accumulate in myeloid progenitors are similar to the those exhibited by aged HSC, including increased frequency and cycling, decreased survival, and diminished per-cell repopulating potential[22]. These observations suggest that age-related defects intrinsic to myeloid progenitors may be the result of intrinsic changes in old HSC. However, whether old HSC and CMP share common molecular changes[59] remains to be determined.

While the detrimental effects of advancing age and decreased lymphocyte production on the adaptive immune response have been well documented[23], the consequences of aging on the innate immune system remain largely unexplored[60]. The innate immune system is a critical first responder to infection. Despite defects in old myeloid progenitors, their increased number may compensate for the fact that they do not produce progeny as efficiently as their young counterparts, which results in a relatively normal number of mature myeloid cells. However, reports of diminished function of aged neutrophils[61], macrophages[62], and dendritic cells[63] indicates that the innate immune response may become significantly compromised with age. Consequently, the unexpected observation that myeloid progenitors from old mice are intrinsically defective indicates that age-related defects may contribute the overall reduction in myeloid cell function in the elderly.

While this study used CML as a model with which to investigate the larger question of how aging affects leukemia development, the data nevertheless provide new insights into this disease. First, the demonstration that the degree of lymphoid disease

mediated by BCR-ABL^{P210} is related to overall levels of B lymphopoiesis provides an explanation for the paradoxical observation that while CML is considered a stem cell disease, it presents as a MPD with relatively rare lymphoid involvement. Second, age-related defects in myeloid progenitors may explain in part why human CML presents with a chronic rather than an acute course[64]. Finally, we demonstrate that patterns of lymphoid and myeloid disease exhibited in primary recipients of young and old BM$^{BCR-ABL}$ are conserved following transplantation of leukemic cells into secondary recipients. This finding suggests an intrinsic role for senescence in governing the behavior of LSC and demonstrates the impact of aging on disease development.

Acknowledgements

This work was supported by grants from the National Institutes of Health (AG-21459) and the U.S. Department of Defense (W81XWHO410795). O.N.W is an investigator of the Howard Hughes Medical Institute. R.A.J.S. is supported by a fellowship from the California Institute for Regenerative Medicine (TI-00005). The UCLA Flow Cytometry Core Facility is supported by grants from the National Institutes of Health (CA-16042, AI-28697).

Author Contributions

The study was designed by R.A.J.S., E.M.-R., O.N.W. and K.D.; experiments were performed and data was analyzed by R.A.J.S. with assistance from E.M.-R.; critical reagents were provided by J.M.; the manuscript was written by R.A.J.S., E.M.-R., and K.D.

References

1. Shizuru JA, Negrin RS, Weissman IL. Hematopoietic stem and progenitor cells: clinical and preclinical regeneration of the hematolymphoid system. Annu Rev Med. 2005;56:509-538.
2. Till JE, McCulloch EA. A direct measurement of the radiation sensitivity of normal mouse bone marrow cells. Radiat Res. 1961;14:213-222.
3. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404:193-197.

4. Igarashi H, Gregory SC, Yokota T, Sakaguchi N, Kincade PW. Transcription from the RAG1 locus marks the earliest lymphocyte progenitors in bone marrow. Immunity. 2002;17:117-130.
5. Kondo M, Weissman IL, Akashi K. Identification of clonogenic common lymphoid progenitors in mouse bone marrow. Cell. 1997;91:661-672.
6. Hardy RR, Carmack CE, Shinton SA, Kemp JD, Hayakawa K. Resolution and characterization of pro-B and pre-pro-B cell stages in normal mouse bone marrow. J Exp Med. 1991;173:1213-1225.
7. Hirose J, Kouro T, Igarashi H, Yokota T, Sakaguchi N, Kincade PW. A developing picture of lymphopoiesis in bone marrow. Immunol Rev. 2002;189:28-40.
8. Montecino-Rodriguez E, Dorshkind K. Evolving patterns of lymphopoiesis from embryogenesis through senescence. Immunity. 2006;24:659-662.
9. Allman D, Miller JP. The aging of early B-cell precursors. Immunol Rev. 2005;205:18-29.
10. Miller JP, Allman D. The decline in B lymphopoiesis in aged mice reflects loss of very early B-lineage precursors. J Immunol. 2003;171:2326-2330.
11. Min H, Montecino-Rodriguez E, Dorshkind K. Effects of aging on the common lymphoid progenitor to pro-B cell transition. J Immunol. 2006;176:1007-1012.
12. Stephan RP, Sanders VM, Witte PL. Stage-specific alterations in murine B lymphopoiesis with age. Int Immunol. 1996;8:509-518.
13. Riley RL, Van der Put E, King AM, Frasca D, Blomberg BB. Deficient B lymphopoiesis in murine senescence: potential roles for dysregulation of E2A, Pax-5, and STAT5. Semin Immunol. 2005;17:330-336.
14. Cambier J. Immunosenescence: a problem of lymphopoiesis, homeostasis, microenvironment, and signaling. Immunol Rev. 2005;205:5-6.
15. Chen J, Astle CM, Harrison DE. Development and aging of primitive hematopoietic stem cells in BALB/cBy mice. Experimental Hematology. 1999;27:928-935.
16. Ogden DA, Mickliem HS. The fate of serially transplanted bone marrow cell populations from young and old donors. Transplantation. 1976;22:287-293.
17. Morrison SJ, Wandycz AM, Akashi K, Globerson A, Weissman IL. The aging of hematopoietic stem cells. Nat Med. 1996;2:1011-1016.
18. Liang Y, Van Zant G, Szilvassy SJ. Effects of aging on the homing and engraftment of murine hematopoietic stem and progenitor cells. Blood. 2005;106:1479-1487.
19. Rossi DJ, Bryder D, Zahn JM, et al. Cell intrinsic alterations underlie hematopoietic stem cell aging. Proc Natl Acad Sci U S A. 2005;102:9194-9199.
20. Sudo K, Ema H, Morita Y, Nakauchi H. Age-associated Characteristics of Murine Hematopoietic Stem Cells. J Exp Med. 2000;192:1273-1280.
21. Min H, Montecino-Rodriguez E, Dorshkind K. Effects of aging on early B- and T-cell development. Immunological Reviews. 2005;205:7-17.
22. Geiger H, Van Zant G. The aging of lympho-hematopoietic stem cells. Nat Immunol. 2002;3:329-333.
23. Linton PJ, Dorshkind K. Age-related changes in lymphocyte development and function. Nat Immunol. 2004;5:133-139.

24. Signer RAJ, Montecino-Rodriguez E, Dorshkind K. Aging, B Lymphopoiesis, and Patterns of Leukemogenesis. Exp Geront. 2006:doi:10.1016/j.exger.2006.1011.1010.
25. Passegue E. Hematopoietic stem cells, leukemic stem cells and chronic myelogenous leukemia. Cell Cycle. 2005;4:266-268.
26. Shepherd P, Suffolk R, Halsey J, Allan N. Analysis of molecular breakpoint and m-RNA transcripts in a prospective randomized trial of interferon in chronic myeloid leukaemia: no correlation with clinical features, cytogenetic response, duration of chronic phase, or survival. Br J Haematol. 1995;89:546-554.
27. Nowell PC, Hungerford DA. A minute chromosome in human chronic granulocytic leukemia. Science. 1960;132:1497.
28. Rowley JD. Letter: A new consistent chromosomal abnormality in chronic myelogenous leukaemia identified by quinacrine fluorescence and Giemsa staining. Nature. 1973;243:290-293.
29. Shtivelman E, Lifshitz B, Gale RP, Canaani E. Fused transcript of abl and bcr genes in chronic myelogenous leukaemia. Nature. 1985;315:550-554.
30. Ben-Neriah Y, Daley GQ, Mes-Masson AM, Witte ON, Baltimore D. The chronic myelogenous leukemia-specific P210 protein is the product of the bcr/abl hybrid gene. Science. 1986;233:212-214.
31. Stam K, Heisterkamp N, Grosveld G, et al. Evidence of a new chimeric bcr/c-abl mRNA in patients with chronic myelocytic leukemia and the Philadelphia chromosome. N Engl J Med. 1985;313:1429-1433.
32. Wong S, Witte ON. The BCR-ABL story: bench to bedside and back. Annu Rev Immunol. 2004;22:247-306.
33. Fialkow PJ, Gartler SM, Yoshida A. Clonal origin of chronic myelocytic leukemia in man. Proc Natl Acad Sci U S A. 1967;58:1468-1471.
34. Fialkow PJ, Jacobson RJ, Papayannopoulou T. Chronic myelocytic leukemia: clonal origin in a stem cell common to the granulocyte, erythrocyte, platelet and monocyte/macrophage. Am J Med. 1977;63:125-130.
35. Takahashi N, Miura I, Saitoh K, Miura AB. Lineage involvement of stem cells bearing the philadelphia chromosome in chronic myeloid leukemia in the chronic phase as shown by a combination of fluorescence-activated cell sorting and fluorescence in situ hybridization. Blood. 1998;92:4758-4763.
36. Castor A, Nilsson L, Astrand-Grundstrom I, et al. Distinct patterns of hematopoietic stem cell involvement in acute lymphoblastic leukemia. Nat Med. 2005;11:630-637.
37. Kabarowski JH, Witte ON. Consequences of BCR-ABL expression within the hematopoietic stem cell in chronic myeloid leukemia. Stem Cells. 2000;18:399-408.
38. Spangrude GJ, Heimfeld S, Weissman IL. Purification and characterization of mouse hematopoietic stem cells. Science. 1988;241:58-62.
39. Michor F, Iwasa Y, Nowak MA. The age incidence of chronic myeloid leukemia can be explained by a one-mutation model. PNAS. 2006;103:14931-14934.
40. Hawley RG, Lieu FH, Fong AZ, Hawley TS. Versatile retroviral vectors for potential use in gene therapy. Gene Ther. 1994;1:136-138.
41. Muller AJ, Young JC, Pendergast AM, et al. BCR first exon sequences specifically activate the BCR/ABL tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. Mol Cell Biol. 1991;11:1785-1792.

42. Allman D, Li J, Hardy RR. Commitment to the B lymphoid lineage occurs before DH-JH recombination. J Exp Med. 1999;189:735-740.
43. Collins LS, Dorshkind K. A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. J Immunol. 1987;138:1082-1087.
44. Whitlock CA, Witte ON. Long-term culture of B lymphocytes and their precursors from murine bone marrow. Proc Natl Acad Sci U S A. 1982;79:3608-3612.
45. Wong S, Witte ON. Modeling Philadelphia chromosome positive leukemias. Oncogene. 2001;20:5644-5659.
46. Mombaerts P, Iacomini J, Johnson RS, Herrup K, Tonegawa S, Papaioannou VE. RAG-1-deficient mice have no mature B and T lymphocytes. Cell. 1992;68:869-877.
47. Bhattacharya D, Rossi DJ, Bryder D, Weissman IL. Purified hematopoietic stem cell engraftment of rare niches corrects severe lymphoid deficiencies without host conditioning. J Exp Med. 2006;203:73-85.
48. Lim YM, Wong S, Lau G, Witte ON, Colicelli J. BCR/ABL inhibition by an escort/phosphatase fusion protein. Proc Natl Acad Sci U S A. 2000;97:12233-12238.
49. Jamieson CH, Ailles LE, Dylla SJ, et al. Granulocyte-macrophage progenitors as candidate leukemic stem cells in blast-crisis CML. N Engl J Med. 2004;351:657-667.
50. Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. Nat Med. 1997;3:730-737.
51. Lapidot T, Sirard C, Vormoor J, et al. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. Nature. 1994;367:645-648.
52. Huntly BJ, Shigematsu H, Deguchi K, et al. MOZ-TIF2, but not BCR-ABL, confers properties of leukemic stem cells to committed murine hematopoietic progenitors. Cancer Cell. 2004;6:587-596.
53. Jaiswal S, Traver D, Miyamoto T, Akashi K, Lagasse E, Weissman IL. Expression of BCR/ABL and BCL-2 in myeloid progenitors leads to myeloid leukemias. Proc Natl Acad Sci U S A. 2003;100:10002-10007.
54. Huettner CS, Zhang P, Van Etten RA, Tenen DG. Reversibility of acute B-cell leukaemia induced by BCR-ABL1. Nat Genet. 2000;24:57-60.
55. Hu Y, Swerdlow S, Duffy TM, Weinmann R, Lee FY, Li S. Targeting multiple kinase pathways in leukemic progenitors and stem cells is essential for improved treatment of Ph+ leukemia in mice. PNAS. 2006:0606509103.
56. Krause DS, Lazarides K, von Andrian UH, Van Etten RA. Requirement for CD44 in homing and engraftment of BCR-ABL-expressing leukemic stem cells. Nat Med. 2006;12:1175-1180.
57. Jung D, Giallourakis C, Mostoslavsky R, Alt FW. Mechanism and control of V(D)J recombination at the immunoglobulin heavy chain locus. Annu Rev Immunol. 2006;24:541-570.
58. Greaves MF, Wiemels J. Origins of chromosome translocations in childhood leukaemia. Nat Rev Cancer. 2003;3:639-649.
59. Janzen V, Forkert R, Fleming HE, et al. Stem-cell ageing modified by the cyclin-dependent kinase inhibitor p16INK4a. Nature. 2006;443:421-426.
60. Plackett TP, Boehmer ED, Faunce DE, Kovacs EJ. Aging and innate immune cells. J Leukoc Biol. 2004;76:291-299.

61. Fu YK, Arkins S, Li YM, Dantzer R, Kelley KW. Reduction in superoxide anion secretion and bactericidal activity of neutrophils from aged rats: reversal by the combination of gamma interferon and growth hormone. Infect Immun. 1994;62:1-8.
62. Stout RD, Suttles J. Immunosenescence and macrophage functional plasticity: dysregulation of macrophage function by age-associated microenvironmental changes. Immunol Rev. 2005;205:60-71.
63. Komatsubara S, Cinader B, Muramatsu S. Polymorphism of age-related changes in stimulatory capacity of murine dendritic cells. Mech Ageing Dev. 1986;37:163-173.
64. Li S, Gillessen S, Tomasson MH, Dranoff G, Gilliland DG, Van Etten RA. Interleukin 3 and granulocyte-macrophage colony-stimulating factor are not required for induction of chronic myeloid leukemia-like myeloproliferative disease in mice by BCR/ABL. Blood. 2001;97:1442-1450.

Figure 1. Age alters the phenotype of BCR-ABL^{P210} induced leukemia.
(A) BM from young or old B6 mice was harvested on day 8 following 5-FU treatment, infected with a retrovirus carrying a bicistronic IRES expression vector encoding BCR-ABL^{P210} and a reporter EGFP gene, and transplanted into sublethally irradiated, young syngeneic or congenic recipients. Control recipients received BM transduced with EGFP alone. Leukemias are accompanied by decreased BM (B) and increased splenic (C) cellularity. Cell numbers represent mean values ± SEM obtained from 5 independent experiments with 29 recipients of young BM$^{BCR-ABL}$, 24 recipients of old BM$^{BCR-ABL}$, 9 recipients of young BM^{EGFP}, and 5 recipients of old BM^{EGFP}. (D) Leukemic cells in the BM were characterized by flow cytometry for expression of GFP (BCR-ABL^{P210}) in combination with lineage specific cell surface antigens. Examples of recipients with a MPD (upper panels), B lymphoid leukemia (middle panels), and both a MPD and lymphoid leukemia (lower panels) are shown. Recipients that developed a MPD had increased granulocytes and decreased lymphocytes in the spleen (upper right panel), while the ones that developed B lymphoid leukemia had increased lymphoid cells and blasts in the spleen (middle right panel) compared to controls. Cytospin preparations of spleen cells were visualized following wright giemsa staining and visualized at 400x magnification. Summary of the incidence of leukemia by phenotype in B6 (E) and *Rag1*$^{-/-}$ (F) recipients of young and old BM$^{BCR-ABL}$ cells. Data in (E) are based on the same recipients as in (B) and (C). Data in (F) are based on 8 recipients of young and 7 recipients of old BM$^{BCR-ABL}$ cells.

Figure 2. Age-related B lymphopoietic defects diminish the leukemogenic potential of BCR-ABL^{P210} transformed BM cells.
(A) Immunostaining used to define pre-pro-B cells (Lin$^-$CD19$^-$CD45R$^+$Ly6-c$^-$AA4.1$^+$) and pro/pre-B cells (Lin$^-$CD19$^+$CD45R$^+$Ly6-c$^-$AA4.1$^+$) in murine BM. (B) The frequency of lymphoid progenitor populations in the BM progressively declines in mice of increasing age. Groups of young (5-7 weeks; n=4), middle-age (42-44 weeks; n=2) and old (90-104 weeks; n=3) mice were analyzed. Steady state frequencies are presented as the mean ± SEM, and 2 pooled middle-age mice. 5-FU frequencies are presented from the pooled BM of 4 young, 7 middle-age, and 4 old mice. Total B lineage cells is all

$CD19^+CD45R^+$ cells. Pre-pro B cell frequencies are 0.103% in young, 0.073% in middle-age, and 0.01% in old 5-FU treated mice, respectively. (C) The incidence of B lymphoid leukemia in *Rag1*$^{-/-}$ recipients of $BM^{BCR\text{-}ABL}$ cells is reduced with increasing BM age. Recipients of middle-age (n=8) $BM^{BCR\text{-}ABL}$ cells develop B lymphoid leukemia less frequently than recipients of young (n=8) $BM^{BCR\text{-}ABL}$ cells, but more frequently than recipients of old (n=7) $BM^{BCR\text{-}ABL}$ cells. The recipients of young and old $BM^{BCR\text{-}ABL}$ are the same as shown in Figure 1F. (D) 3 x 10^5 Young and old BM cells transduced with EGFP (left panel) or BCR-ABL^{P210} (right panel) were used to establish hematopoietic cultures on confluent S17 stromal layers in conditions optimized for the long-term growth of B lineage cells[44]. The number of B lineage cells in cultures initiated with young BM^{EGFP} cells is 3-4 fold higher than in those initiated with old BM^{EGFP} cells at week 3 post culture initiation. The number of B lineage cells in cultures initiated with young $BM^{BCR\text{-}ABL}$ cells is approximately 100 fold higher than in those initiated with old $BM^{BCR\text{-}ABL}$ cells at week 3 post culture initiation. Results show that BCR-ABL^{P210} expression increased the growth of young B lineage cells approximately 70 fold, but does not rescue age-related proliferative defects in aged hematopoietic cells. (E) Cultures derived from young $BM^{BCR\text{-}ABL}$ established larger colonies with increased cellularity and a higher frequency of GFP^+ B lineage cells compared to those initiated from old $BM^{BCR\text{-}ABL}$ which produced colonies with a myeloid appearance.

Figure 3. MPDs derived from old $BM^{BCR\text{-}ABL}$ are characterized by a reduced tumor burden.

The frequency of total leukemic GFP^+ (BCR-ABL^+) cells and leukemic myeloid $GFP^+Gr\text{-}1^+CD11b^+$ cells in the BM (A) and spleen (B) of recipients of young $BM^{BCR\text{-}ABL}$ cells is increased compared to recipients of old $BM^{BCR\text{-}ABL}$ cells. (C) Decreased B lineage cells in the spleen of young $BM^{BCR\text{-}ABL}$ recipients compared to old $BM^{BCR\text{-}ABL}$ recipients. Cell frequency in (A-C) is presented as the mean frequency of cells ± SEM from 17 recipients of young and 23 recipients of old $BM^{BCR\text{-}ABL}$ that developed MPDs analyzed in 5 independent experiments. (D) Immunostaining used to define populations enriched for leukemic HSC ($GFP^+Lin^-Sca\text{-}1^{Hi}CD117^{Hi}$) CMP ($GFP^+Lin^-Sca\text{-}1^-CD127^-CD16/32^{+/Lo}CD117^{Hi}$), and GMP ($GFP^+Lin^-Sca\text{-}1^-CD127^-CD16/32^{+/Lo}CD117^{Hi}$) in the

BM and spleen of $BM^{BCR-ABL}$ recipients. (E) Recipients of young $BM^{BCR-ABL}$ cells have more leukemic (GFP^+) HSC and myeloid progenitors (CMP and GMP) in their BM (upper panel) and spleen (lower panel) compared to those transplanted with old $BM^{BCR-ABL}$ cells. The frequency of GFP^+ HSC and myeloid progenitors is presented as the mean ± SEM of 12 recipients of young and 13 recipients of old $BM^{BCR-ABL}$ that developed MPDs with no B lineage involvement.

Figure 4. Myelopoietic defects are present in old mice.

(A) The frequency of CMP and GMP are increased in the BM of old (78-105 weeks) compared to young (5 weeks) mice both at steady state and at day 8 post 5-FU treatment. Steady state frequencies are presented as the mean ± SEM of 16 young and 12 old mice analyzed in 4 independent experiments. 5-FU frequencies are presented from the pooled BM of 9 young and 7 old mice analyzed in two independent experiments. (B) The number of Annexin V^+ CMP and GMP is increased in the BM of old compared to young mice. Cell numbers are presented as the mean ± SEM of 12 young and 8 old mice analyzed in 3 independent experiments. Myeloid colonies derived from whole BM cells (C) and sorted CMP (D) isolated from young mice contain more cells than those derived from BM and CMP isolated from old mice. Cell numbers in (C) are presented as the mean ± SEM of 24 colonies derived from young BM cells and 24 colonies derived from old BM cells picked in 2 independent experiments. Cell numbers in (D) are presented as the mean of 12 colonies derived from young CMP and 12 colonies derived from old CMP ± SEM picked in 1 of 2 representative experiments. (E) 1.5×10^5 BM cells and 2×10^3 CMP (F) plated in liquid culture supplemented with myelopoietic cytokines isolated from young mice produce more $Gr\text{-}1^+CD11b^+$ myeloid cells compared to their old counterparts. Numbers are presented as the mean ± SEM of 3 to 6 wells analyzed in 1 of 2 representative experiments.

Figure 5. Age-related hematopoietic defects alter the leukemogenic potential of LSC.

(A) Secondary recipients of splenocytes from leukemic mice grafted with young $BM^{BCR-ABL}$ cells developed MPDs and B lymphoid leukemia. 5×10^6 splenocytes from 4 primary

young $BM^{BCR-ABL}$ recipients were transplanted into 3 or 4 secondary recipients each (15 total). (B) Secondary recipients of splenocytes from leukemic mice grafted with old $BM^{BCR-ABL}$ cells develop MPDs with no significant involvement of B lineage cells. 5 x 10^6 splenocytes from 3 primary old $BM^{BCR-ABL}$ recipients were transplanted into 4 secondary recipients each (12 total). (C) The frequency of total leukemic GFP^+ cells and leukemic myeloid $GFP^+Gr\text{-}1^+CD11b^+$ cells in the spleens of secondary recipients of splenocytes from tumors derived from young $BM^{BCR-ABL}$ cells is increased compared to secondary recipients of tumors derived old $BM^{BCR-ABL}$ cells. (D) The frequency of B lineage cells in the spleen of secondary recipients of tumors derived from young $BM^{BCR-ABL}$ cells is decreased compared to secondary recipients of tumors derived from old $BM^{BCR-ABL}$ cells. Cell frequency in (C-D) is presented as the mean frequency of cells ± SEM from 6 secondary recipients of tumors derived from young $BM^{BCR-ABL}$ cells and 12 secondary recipients of tumors derived from old $BM^{BCR-ABL}$ cells. The mice analyzed in (C-D) developed MPDs with no B lineage involvement.

Figure 1

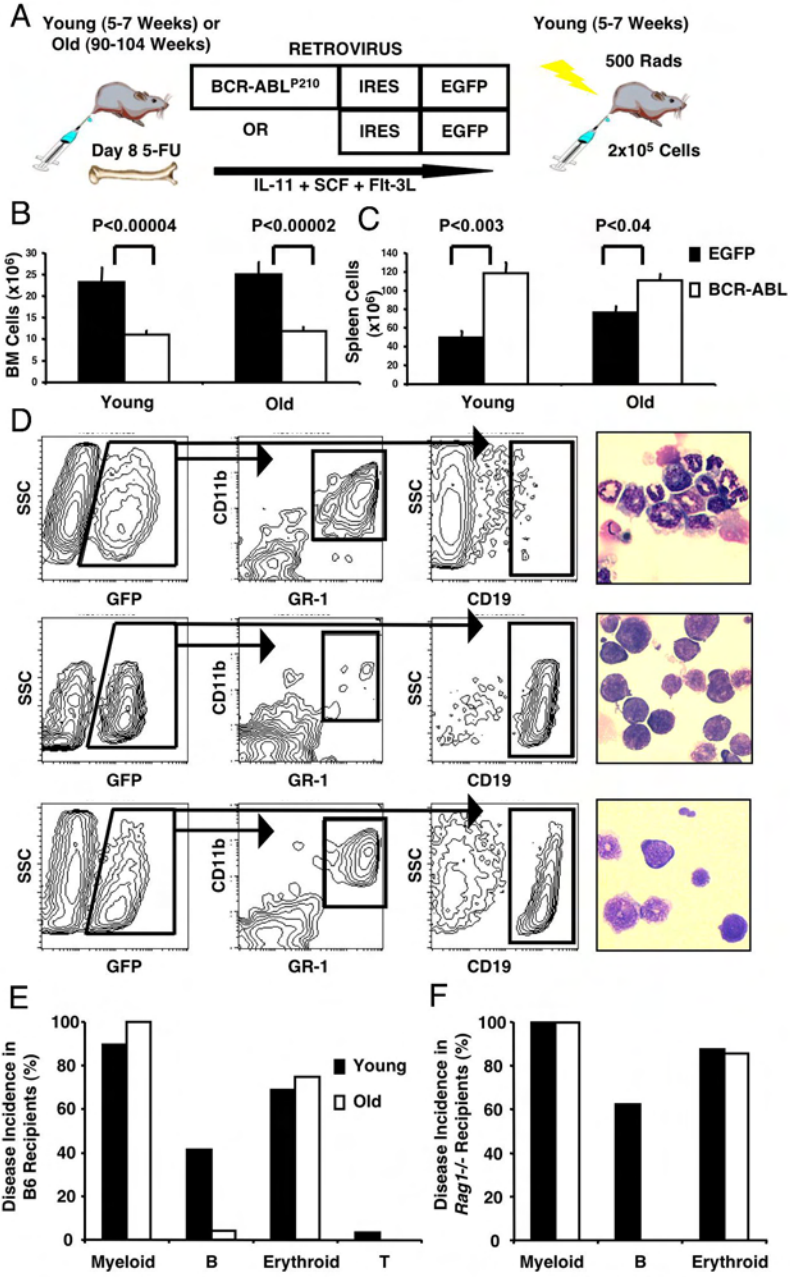
A
Young (5-7 Weeks) or Old (90-104 Weeks)
RETROVIRUS
BCR-ABL P210
IRES
EGFP
OR
IRES
EGFP
Young (5-7 Weeks)
500 Rads
Day 8 5-FU
2x10^5 Cells
IL-11 + SCF + Flt-3L
B
P<0.00004
P<0.00002
BM Cells (x10^6)
Young
Old
C
P<0.003
P<0.04
Spleen Cells (x10^6)
EGFP
BCR-ABL
Young
Old
D
SSC
GFP
CD11b
GR-1
SSC
CD19
E
Disease Incidence in B6 Recipients (%)
Young
Old
Myeloid
B
Erythroid
T
F
Disease Incidence in Rag1-/- Recipients (%)
Myeloid
B
Erythroid

Figure 2

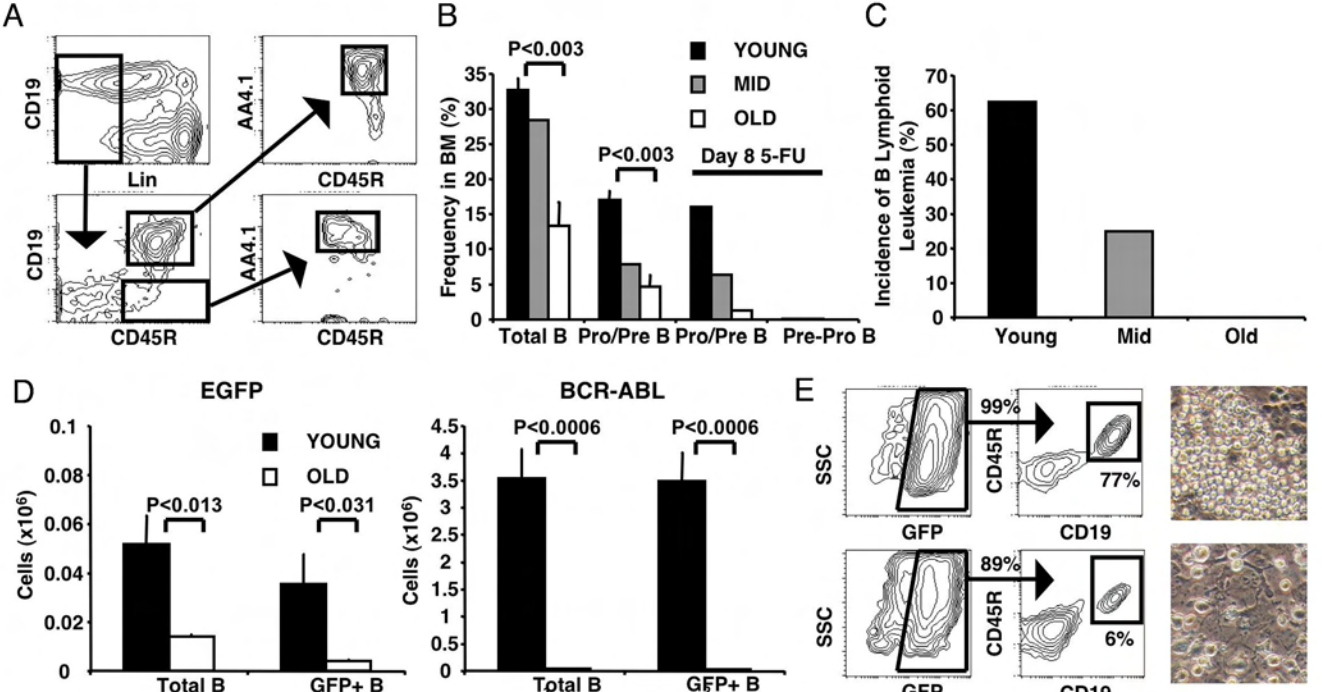
A
CD19
Lin
AA4.1
CD45R
CD19
CD45R
AA4.1
CD45R
B
P<0.003
YOUNG
MID
OLD
Frequency in BM (%)
P<0.003
Day 8 5-FU
Total B
Pro/Pre B
Pro/Pre B
Pre-Pro B
C
Incidence of B Lymphoid Leukemia (%)
Young
Mid
Old
D
EGFP
YOUNG
OLD
Cells (x10^6)
P<0.013
P<0.031
Total B
GFP+ B
BCR-ABL
P<0.0006
P<0.0006
Cells (x10^6)
Total B
GFP+ B
E
SSC
GFP
99%
CD45R
77%
CD19
SSC
GFP
89%
CD45R
6%
CD19

Figure 3

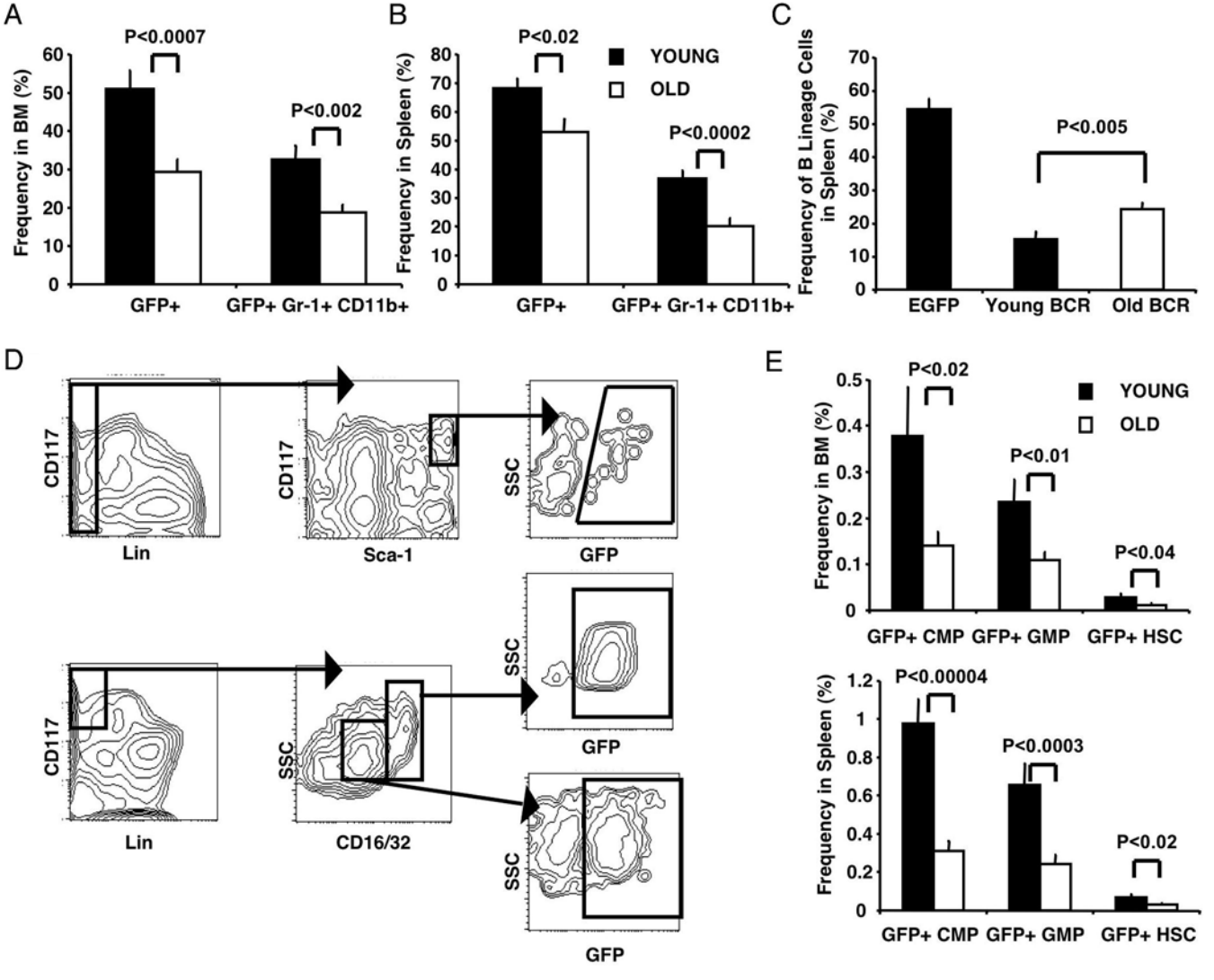
A
P<0.0007
P<0.002
Frequency in BM (%)
GFP+
GFP+ Gr-1+ CD11b+
B
P<0.02
P<0.0002
YOUNG
OLD
Frequency in Spleen (%)
GFP+
GFP+ Gr-1+ CD11b+
C
P<0.005
Frequency of B Lineage Cells in Spleen (%)
EGFP
Young BCR
Old BCR
D
CD117
Lin
CD117
Sca-1
SSC
GFP
SSC
GFP
CD117
Lin
SSC
CD16/32
SSC
GFP
E
P<0.02
P<0.01
P<0.04
YOUNG
OLD
Frequency in BM (%)
GFP+ CMP
GFP+ GMP
GFP+ HSC
P<0.00004
P<0.0003
P<0.02
Frequency in Spleen (%)
GFP+ CMP
GFP+ GMP
GFP+ HSC

Figure 4

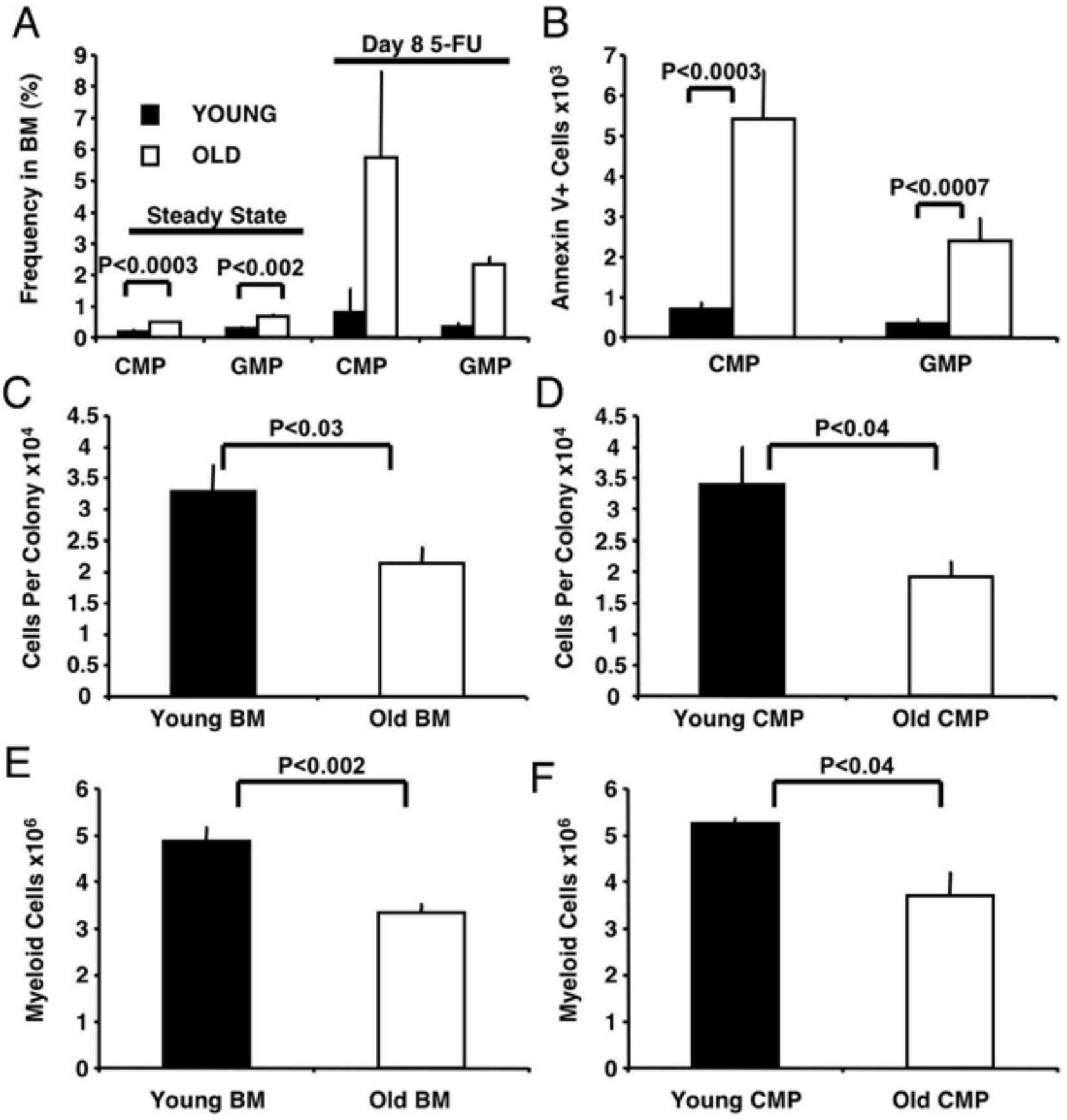
A
Frequency in BM (%)
YOUNG
OLD
Steady State
Day 8 5-FU
P<0.0003
P<0.002
CMP
GMP
CMP
GMP
B
Annexin V+ Cells x10³
P<0.0003
P<0.0007
CMP
GMP
C
Cells Per Colony x10⁴
P<0.03
Young BM
Old BM
D
Cells Per Colony x10⁴
P<0.04
Young CMP
Old CMP
E
Myeloid Cells x10⁶
P<0.002
Young BM
Old BM
F
Myeloid Cells x10⁶
P<0.04
Young CMP
Old CMP

Figure 5

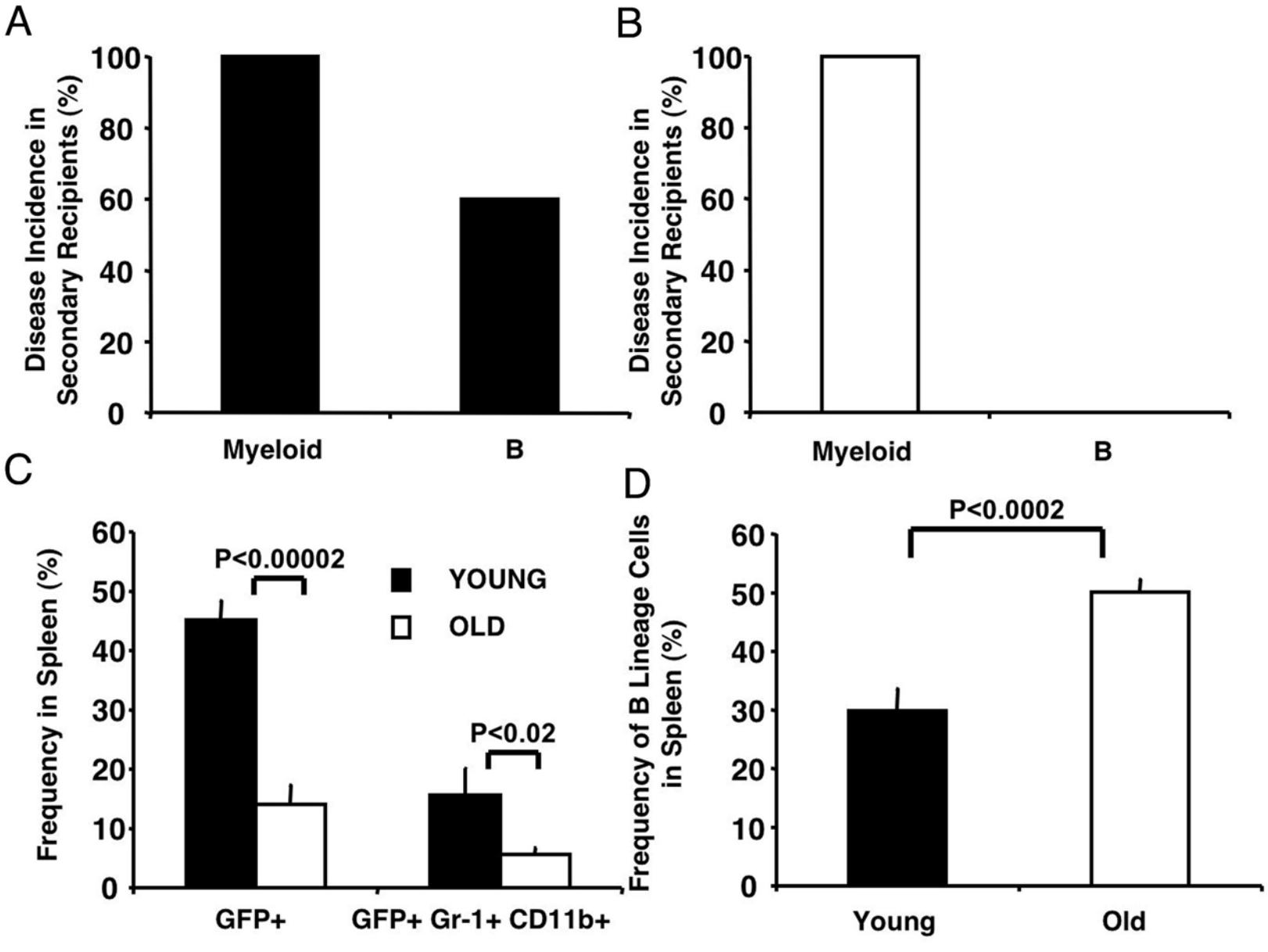
A
Disease Incidence in Secondary Recipients (%)
100
80
60
40
20
0
Myeloid
B
B
Disease Incidence in Secondary Recipients (%)
100
80
60
40
20
0
Myeloid
B
C
Frequency in Spleen (%)
60
50
40
30
20
10
0
P<0.00002
YOUNG
OLD
P<0.02
GFP+
GFP+ Gr-1+ CD11b+
D
Frequency of B Lineage Cells in Spleen (%)
P<0.0002
60
50
40
30
20
10
0
Young
Old

Table 1. Disease Characteristics of Mice Transplanted with Young or Old EGFP or BCR-ABL^{P210} Transuced BM Cells

	BM				Spleen				Disease
Donor	Total Cells x10^6*	%GFP$^+$	%GFP$^+$ Gr-1$^+$ CD11b$^+$	%GFP$^+$ CD45R$^+$ CD19$^+$	Total Cells x10^6	%GFP$^+$	%GFP$^+$ Gr-1$^+$ CD11b$^+$	%GFP$^+$ CD45R$^+$ CD19$^+$	Myeloid (M) or Lymphoid (B)
Young									
EGFP1	9.4	1.9	1.1	0.6	15.8	5.9	2.8	0.9	None
EGFP2	26	1.5	0.8	0.2	52.8	1.8	0.5	1.3	None
EGFP3	13.2	2.1	0.9	0.3	48	2.5	0.4	1.7	None
EGFP4	18.8	1.9	0.9	0.3	61.6	5.3	0.3	1.5	None
BCR1	9.2	62.0	39.1	0.2	52.8	75.3	55.1	1.0	M
BCR2	N/A	64.1	12.7	2.5	N/A	84.8	16.5	1.3	M
BCR3	11	76.3	52.3	0.3	90.2	86.7	42.8	0.9	M
BCR4	10.2	65.4	25.7	0.1	92.2	76.6	50	0.7	M
BCR5	12.0	55.7	44.7	5.2	108.5	66.0	38.9	6.4	M + B
BCR6	7.2	32.0	10.9	19.5	130.4	74.5	9.9	15.1	M + B
BCR7	11.6	60.7	1.3	58.8	56.8	58.6	5.2	48.4	B
BCR8	10.2	82.0	56.9	0.5	199.2	56.0	37.6	2.9	M
Old									
EGFP1	17.8	2.4	1.7	0.1	72.0	1.2	0.7	0.4	None
EGFP2	23.4	1.4	1.0	0.2	82.6	2.2	1.4	0.3	None
BCR1	8.8	68.9	27.5	0.2	148.8	65.6	18.2	2.6	M
BCR2	20.0	17.8	12.9	0.1	136.3	43.7	12.8	1.2	M
BCR3	15.6	34.4	26.6	0.2	144.0	58.4	32.2	1.7	M
BCR4	9.4	56.0	23.1	0.1	168.0	53.8	21	2.2	M
BCR5	15.2	45.4	24.0	0.1	178.6	62.4	19.5	1.2	M
BCR6	14.8	30.7	25.2	0.1	121.0	50.7	26.5	1.8	M
BCR7	20.6	22.7	18.4	0.1	136.3	52.6	20.3	1.3	M
BCR8	20.8	2.2	1.6	0.1	67.2	21.7	9	0.7	M

*Numbers of BM cells are from 2 femurs and 2 tibias.